Removal of Fibroblasts from Human Epithelial Cell Cultures with Use of a Complement Fixing Monoclonal Antibody Reactive with Human Fibroblasts and Monocytes/Macrophages

Kay H. Singer, Ph.D., Richard M. Scearce, B.S., Debbi T. Tuck, B.S., Leona P. Whichard, M.S., Stephen M. Denning, M.D., and Barton F. Haynes, M.D.

Division of Rheumatology and Immunology (KHS, RMS, DTT, LPW, BFH), Division of Cardiology (SMD), Department of Medicine and Division of Immunology (KHS, BFH), Department of Microbiology and Immunology, Duke University, Durham, North Carolina, U.S.A.

A complement fixing IgM monoclonal antibody (1B10) that reacts with surface membrane molecules of human fibroblasts, tissue macrophages, and peripheral monocytes was produced. In Western blot analysis of detergent extracts of cultured human foreskin fibroblasts, antibody 1B10 detected protein bands of Mr 43,000 and 72–80,000. We used the 1B10 antibody with complement to eliminate most 1B10 positive nonepithelial cells from thymic epithelial (TE) cell cultures, thereby allowing us to grow highly enriched populations of human TE cells. *J Invest Dermatol 92:166–170, 1989*

Overgrowth of epithelial cells by fibroblasts has long been an obstacle to cultivation of normal and abnormal epithelial cells. In recent years, technological advances have been made in the in vitro propagation of human thymic and skin epithelial cells, and in generating epithelial cultures relatively free from contaminating fibroblasts [1–4]. The primary methods used to reduce or eliminate fibroblasts from epithelial cultures have included the following: 1) use of mouse 3T3 fibroblast feeder layers (mitomycin C treated or irradiated) [1]; 2) periodic treatment of cultures with EDTA warmed to 37°C and vigorously sprayed over the cells [5]; 3) in the case of skin, separation of epidermis from dermis prior to preparation of a single cell suspension of epidermal cells [6]; and 4) in the case of thymus, propagation in the presence of D-valine [7,8]. In spite of these approaches, fibroblast overgrowth remains a problem for cultivation of tissues such as thymus and breast, where fibroblasts are not easily separated from epithelial cells prior to culture. We report the production and characterization of a murine IgM monoclonal antibody (1B10) that binds to the surface of human fibroblasts and monocytes/macrophages, fixes complement, and has been used successfully to substantially reduce fibroblasts from epithelial cell cultures. Interestingly, antibody 1B10 also reacts with tissue macrophages and peripheral blood monocytes.

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Reprint requests to: Kay H. Singer, Ph.D., Box 2987, Duke University Medical Center, Durham, North Carolina 27710.

Abbreviations:
DME: Dulbecco's Modified Eagle's Medium
EDTA: ethylenediamine tetraacetic acid
FCS: fetal calf serum
HUFF: human foreskin fibroblasts
PBS: phosphate buffered saline
TE: thymic epithelial

MATERIALS AND METHODS

Cells Human neonatal foreskin fibroblast cultures were initiated by an explant technique as described by Sly and Grub [9], and were propagated in Dulbecco's Modified Eagle's Medium (DME) (GIBCO, Grand Island, NY) containing a final concentration of 10% fetal calf serum (DME/FCS-10) (GIBCO). Human thymic epithelial cells were initiated and propagated as previously described [5] in enriched culture medium [10] consisting of a 3:1 (vol/vol) mixture of Dulbecco's Modified Eagle's Medium (DME, GIBCO Labs, Grand Island, NY) and Ham's F12 nutrient mixture (GIBCO) with final concentrations of the following supplements: 5% fetal calf serum (GIBCO or Sigma); 0.4 μg/ml hydrocortisone (Calbiochem-Behring, La Jolla, CA); 20 ng/ml epidermal growth factor (Collaborative Research, Waltham, MA); 10^{-10} M cholera enterotoxin (Schwarz-Mann, Orangeburg, NJ); 5 μg/ml insulin (Sigma); 1.8×10^{-4} M adenine (Sigma); 100 units/ml penicillin, 100 μg/ml Streptomycin and 0.25 μg/ml Amphoteracin B (GIBCO).

To obtain human thymic fibroblasts, we incubated cultures of thymic epithelial cells and fibroblasts briefly with 0.02% EDTA (37°C). EDTA was sprayed over the cultures to detach fibroblasts. The fibroblasts were then transferred to 75 cm^2 tissue culture flasks (Corning Glassworks Corning, NY) and propagated in DME/FCS-10. Human neonatal foreskin epidermal cells were cultured as previously described [6]. The A431 epidermoid carcinoma cell line was obtained from Dr. Joseph DeLarco (Otsuko Pharmaceuticals, Gaithersburg, MD) [11] and grown in DME/FCS-10. The human fibroblast line CRL 1506 was obtained from the American Type Culture Collection (Rockville, MD) and grown in DME/FCS-10. Synovial fibroblast cultures were initiated from synovial tissue obtained following synovectomy in patients with rheumatoid arthritis or osteoarthritis using the explant method of Sly and Grub [9]. Synovial cultures were propagated in enriched culture medium used for epithelial culture. Mononuclear cells were isolated from human

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peripheral blood following Ficoll Hypaque gradient centrifugation.

Cell lines were characterized using a panel of monoclonal antibodies including anti-keratin antibody AE-1 [12], anti-fibroblast antibody TE-7 [13], and anti-monocyte/macrophage antibody LeuM3 [14].

Production of Monoclonal Antibody 1B10 Antibody 1B10 was the product of a lymphocyte hybrid fusion between P3X63/Ag8 murine myeloma cells and BALB/c spleen cells from an animal immunized with cultured human thymic fibroblasts. For each immunization, $3-10 \times 10^6$ fibroblasts were scraped from a culture flask, washed 3× in Dulbecco's PBS, and injected intraperitoneally into female BALB/c mice. Immunizations were performed on days -40, -34, -26, -19, -12, and -2. Polyclonal antisera were collected and screened for cytotoxicity in a ^{51}Cr release assay using human fibroblasts as targets. The antisera were cytotoxic in the presence of rabbit complement. Hybridomas were generated using standard technology [15]. Briefly, on day 0, 2.25×10^8 splenocytes were fused using 50% polyethylene glycol with myeloma cell line P3X63/Ag8 (P3), NS1-Ag4-1 (NS1), and X63.Ag8.653(653) at a 5:1 splenocyte to myeloma ratio. For the P3X63 parent myeloma, 540 wells were seeded. Growth was observed and supernatants screened from 224 of these for a fusion efficiency of 41.5%. For the NS1 parent myeloma, 528 wells were seeded, and 75 grew and were screened for a fusion efficiency of 14.2%. For the 653 parent myeloma, 480 colonies were seeded and 47 grew and were screened for a fusion efficiency of 9.8%.

Supernatants from colonies that grew were screened by indirect immunofluorescence on 4 μm frozen sections of human thymus [16]. The supernatants were analyzed for fibroblast binding patterns (staining of the interlobular septae and vessels), macrophage binding patterns (binding of dendritic shaped cells with lipofuchsin granules, primarily found in the thymic medulla), epithelial patterns (epithelial cells in thymic cortex and medulla including Hassall's bodies), or MHC Class I patterns (entire thymus including thymocytes).

Of 346 colonies screened, 127 gave a binding pattern on thymus sections. Seventy-seven (77) of these were transferred to 24 well culture dishes, grown up, and supernatants screened by immunofluorescence on thymus sections, skin sections, and cytocentrifuge preparations of human foreskin fibroblasts (HUFF) and the ATCC human fibroblast line CRL 1506.

Based on binding patterns revealed in this screen, we selected 14 hybrids to screen for their ability to fix complement and lyse fibroblasts. Supernatants were screened in a complement mediated microcytotoxicity ^{51}Cr-release assay using as targets HUFF, CRL 1506, and the epidermal carcinoma cell line A431. Monoclonal antibody to HLA Class I antigens (3F10) was used as a positive control.

Antibody 1B10 emerged as a likely candidate for the cytotoxic function we were seeking and the hybridoma was cloned by limiting dilution. Hybrids producing 1B10 were readily cloned and maintained stable production of antibody 1B10. They adapted readily to growth in ascites form. 1B10 was isotyped as an IgM using flat bed immunoisoelectric focussing. Ascites fluid from the cloned 1B10 hybrid growing in ascites was screened for cytotoxicity on HUFF and A431 using an antibody and complement mediated ^{51}Cr release assay.

Immunofluorescence and Flow Cytometry Indirect immunofluorescence on cryostat sections of human thymus and skin and a human fibrosarcoma was performed as previously described [16]. Indirect immunofluorescence on acetone fixed cytocentrifuge preparations of cells was performed as previously described. Detection of cell surface antigen expression was by indirect immunofluorescence on viable cell suspensions. Cells were evaluated for positive staining using a Nikon Optiphot fluorescence microscope or an Orthro cytofluorograph.

Cytotoxicity Complement-dependent cytotoxicity mediated by antibody 1B10 was demonstrated in a ^{51}Cr release assay. Cells were

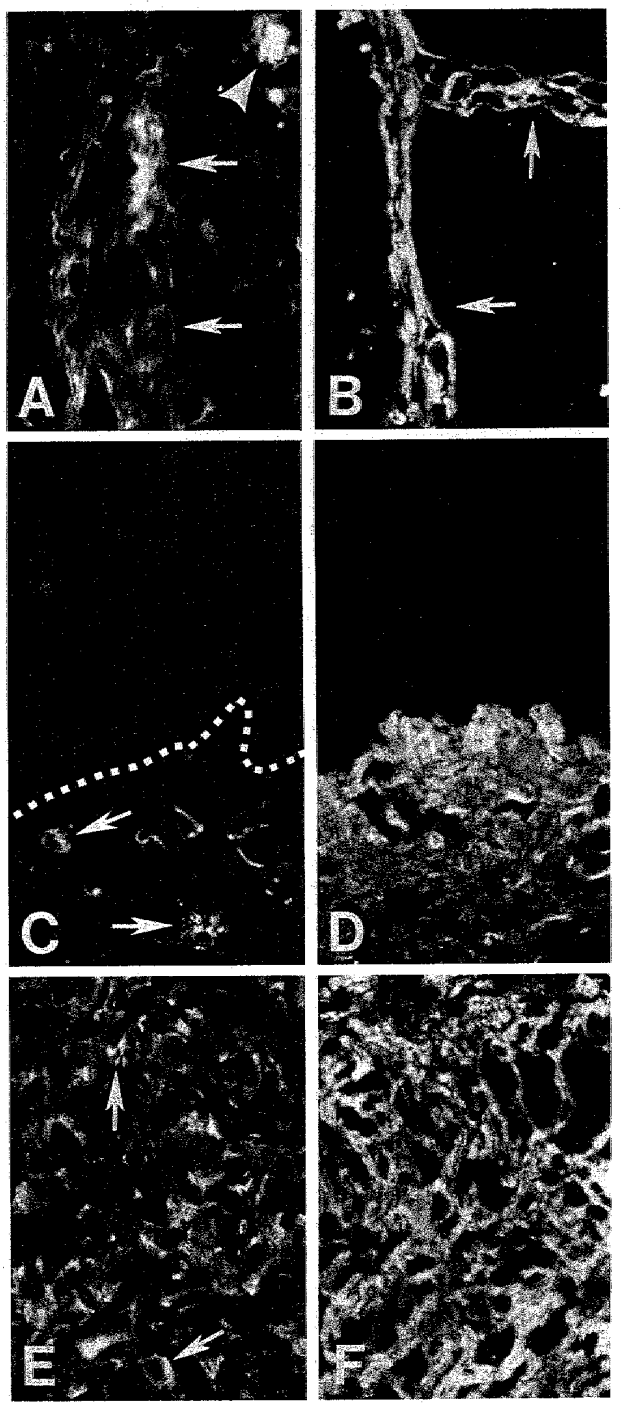

Figure 1. Comparison of the reactivity patterns of antibodies 1B10 and TE-7 on cryostat sections of human thymus and skin and a fibrosarcoma. *A:* 1B10 on thymus. *Arrows* indicate reactivity with cells along interlobular septum. *Arrowhead* indicates reactivity with a macrophage. *B:* TE-7 on human thymus, showing fibrillar staining pattern along interlobular septum. *C:* 1B10 on human skin. *Dashed line* indicates dermoepidermal junction. *Arrows* indicate individual cells reactive with 1B10. *D:* TE-7 on human skin showing fibrillar staining over entire dermis. *E:* 1B10 on fibrosarcoma. *Arrows* indicate reactivity with individual cells. *F:* TE-7 on fibrosarcoma showing fibrillar pattern of reactivity.

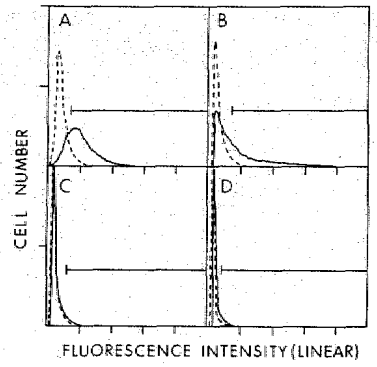

Figure 2. Cytofluorograph histograms showing reactivity of antibody 1B10 *(solid lines)* with *A:* Cultured human foreskin fibroblasts; *B:* cultured human synovial fibroblasts; *C:* cultured human thymic epithelial cells; and *D:* cultured human epidermal keratinocytes. *Broken line* indicates negative control staining with P3 supernatant.

grown on flat bottom 96 well tissue culture plates, incubated overnight with ^{51}Cr-sodium chromate (25 μCi/ml), and washed thoroughly. Antibodies at various dilutions in DME/FCS-10 were added to each well (100 μl/well) and cells incubated 40 min at 37°C. Young rabbit serum (Pel-Freez, Rogers, AZ) was added to each well (100 μl/well) to give a final dilution of 1:4. Cells were incubated 45 min at 37°C. Supernatants were removed and counted in an LKB Rackgamma 1270 (LKB Instruments, Turku, Finland). Total releasable radioactivity was determined by adding 200 μl Triton X-100 (1%) to triplicate wells. Percent cytotoxicity was calculated by the following formula:

$$\% \text{ Specific cytoxicity} = \frac{\text{cpm released in the presence of Ab + C'} - \text{cpm released in the presence of C' alone}}{\text{cpm released in 1\% Triton X-100} - \text{cpm released in presence of C' alone}} \times 100.$$

All samples were assayed in triplicate.

Treatment of Epithelial Cultures with Antibody 1B10 and Complement Primary thymic epithelial cultures that contained fibroblasts were treated with 1B10 antibody (1:800 in DME/FCS-10) for 40 min at 37°C. The cells were rinsed with DME. Young rabbit serum (1:8 in DME/FCS-10) was added to each plate as a source of complement and cells were incubated for 45 min at 37°C. Following this treatment, we vigorously sprayed the cell monolayers with medium to remove dead fibroblasts. In some experiments cell cultures were trypsinized from the dish and treated with antibody and complement while in suspension. Indirect immunofluorescence was then performed on the cells in suspension. Expression of cell surface antigens before and after treatment with antibody and complement was assessed by cytofluorograph analysis.

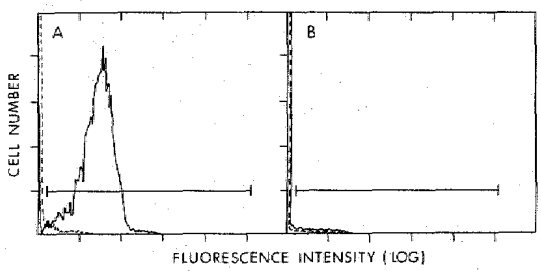

Figure 3. Cytofluorograph histograms showing reactivity of antibody 1B10 *(solid line)* with peripheral blood mononuclear cells determined by selective gate analysis. *A:* Gating on monocyte 1B10 was reactive with 95% of cells. *B:* Gating on lymphocytes 1B10 was reactive with 0% of cells.

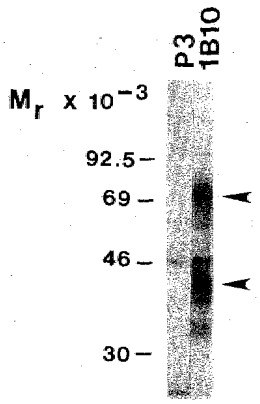

Figure 4. Western blot analysis of detergent extract of human foreskin fibroblasts. Antibody 1B10 specifically detected bands migrating at Mr 43,000 and 72–80,000 *(arrowheads)*. A doublet of Mr 32,000 and 34,000 was also detected; however, the same doublet was frequently seen with the control antibody P3.

SDS-PAGE and Immunoblot Analysis Human foreskin fibroblasts were grown to confluence in 75 cm^2 tissue culture flasks. Cells on 8 flasks were rinsed 3× with PBS, scraped into PBS, and pelleted. The pellet was resuspended in 2 ml of extraction buffer [0.01 M Tris, 0.15 M NaCl, 1% (w/v) Triton X-100, pH 7.2 containing 1 mM phenylmethysulfonyl fluoride (Sigma), 1 mM 0-phenanthroline (Sigma), 1 mM parahydroxy mercuribenzoate (Sigma), 1 mM iodoacetamide (Sigma)], and incubated on ice for 30 min. The extraction mixture was then centrifuged for 15 min in a microfuge (Eppendorf), 27,000 × *g*, and the supernatant containing detergent extracted proteins was subjected to SDS-PAGE on 12.5% gels [17]. Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose and subjected to immunoblot analysis [18].

RESULTS

Tissue Distribution of the Antigen Detected by 1B10 Cryostat sections of human tissues were examined for reactivity with 1B10 antibody by indirect immunofluorescence (Fig 1). The reactivity with 1B10 was compared with that of TE-7 which binds to an intracellular antigen specific for human fibroblasts [13]. On thymus tissue sections antibody 1B10 was bound to the thymic capsule, interlobular septae and to thymic vessels. Thymic macrophages containing lipofuchsin granules were also bound by antibody 1B10. Occasionally, faint cytoplasmic reactivity of thymic epithelial cells was also seen. On skin sections, antibody 1B10 reacted only with fibroblasts and macrophages. Antibody 1B10 did not bind to collagen bundles. As in thymus, faint staining of skin epithelial cells in a granular, cytoplasmic pattern was occasionally present. Antibody 1B10 reacted in a cell surface pattern with malignant cells within a fibrosarcoma tissue.

Because 1B10 faintly stained TE and skin keratinocytes, it was critical to determine the specificity of this reagent with regard to surface reactivity with fibroblasts and epithelial cells. We evaluated a number of cell types for reactivity with 1B10 by indirect immunofluorescence using an Orthro cytofluorograph (Fig 2). Human foreskin fibroblasts (Fig 2*A*) and cultured synovial fibroblasts (Fig 2*B*) expressed the 1B10 antigen on the cell surface. In contrast, neither thymic epithelial cells (Fig 2*C*) nor epidermal keratinocytes (Fig 2*D*) expressed 1B10 on the cell surface. Because we observed binding of antibody 1B10 to macrophages in tissue sections, we evaluated the binding of 1B10 to peripheral blood monocytes and lymphocytes (Fig 3). When monocytes were analyzed separately from

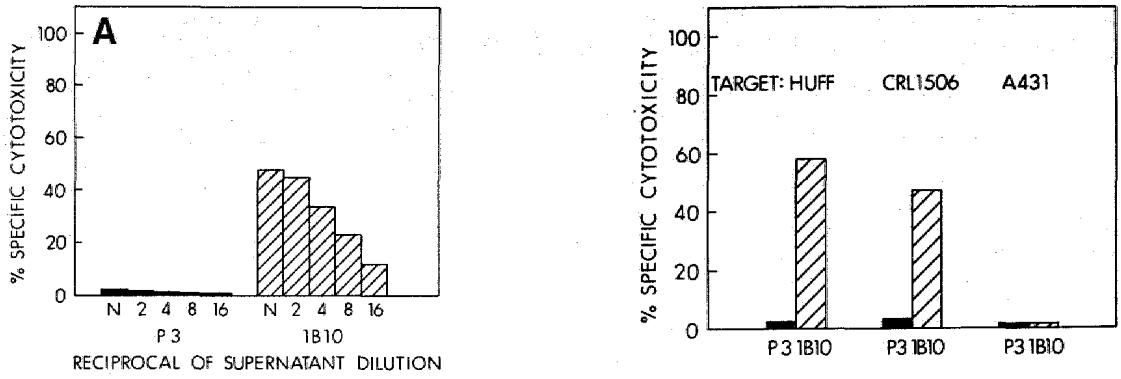

Figure 5. *A:* Lysis of human foreskin fibroblasts by antibody 1B10 and complement. ^{51}Cr-labeled fibroblasts were incubated with supernatant from P3 parent myeloma cultures *(solid bars)* or from 1B10 hybridoma *(hatched bars)*. Young rabbit serum was added as a source of complement and cytotoxicity was assessed as release of ^{51}Cr. *B:* Supernatant from 1B10, clone 4 hybridoma was tested in a complement mediated ^{51}Cr release assay for the ability to lyse human foreskin fibroblasts (HUFF), the fibroblast cell line CRL 1506, and the epidermal cell line A431.

lymphocytes by selective gate analysis, we found that 95% of monocytes were 1B10+ and 0% of lymphocytes were 1B10+. Thus, the antigen detected by antibody 1B10 is expressed on the surface of fibroblasts and peripheral blood monocytes but not on the surface of lymphocytes or epithelial cells.

Western Blot Analysis of the Antigen Detected on Human Foreskin Fibroblasts by Antibody 1B10 In order to characterize the molecule(s) detected on fibroblasts by antibody 1B10, we prepared detergent extracts of cultured human foreskin fibroblasts and subjected the extracts to Western blot analysis using antibody

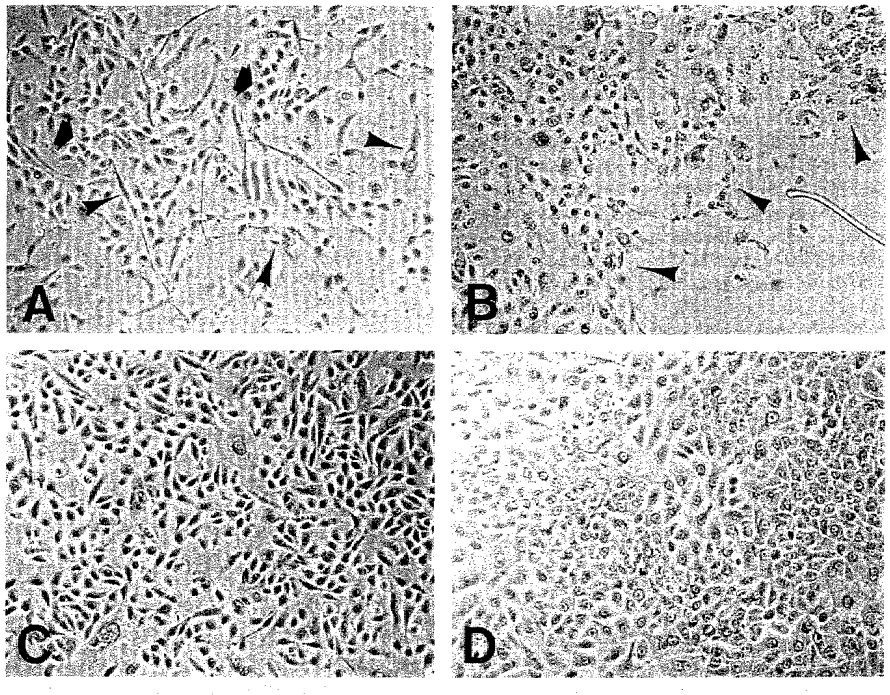

Figure 6. Removal of fibroblasts from mixed cultures of thymic epithelial cells and fibroblasts. A dish containing primary cultures of thymic epithelial cells was treated with antibody 1B10 and complement as described in *Materials and Methods. A:* An area of mixed epithelial cells and fibroblasts prior to treatment. *Arrows* indicate nests of epithelial cells. *Arrowheads* indicate fibroblasts. *B:* An area of lysed fibroblasts immediately following treatment with antibody 1B10 and complement. *C:* Epithelial cells remaining on the dish immediately following treatment with antibody 1B10 and complement. *D:* The same dish of epithelial cells 7 d following treatment with antibody 1B10 and complement (×400).

1B10 supernatant and P3 supernatant as a negative control. As shown in Fig 6, 1B10 specifically detected bands migrating at Mr 43,000 and 72–80,000.

Complement-Mediated Cytotoxic Activity of Antibody 1B10 The hybridoma secreting 1B10 antibody was originally selected by a screen for cytotoxic activity on human foreskin fibroblasts in the presence of complement. Supernatant from the hybridoma was titrated to determine cytotoxic activity against human foreskin fibroblasts (Fig 5*A*). The hybridoma was then cloned and the clones were screened for complement-mediated cytotoxic activity on foreskin fibroblasts, the fibroblast cell line CRL 1506, and the epithelial carcinoma cell line A431 in a ^{51}Cr-release assay (Fig 5*B*). Antibody produced by 1B10 clone 4 lysed both HUFF and CRL 1506 but did not lyse A431 epithelial cells.

Use of Antibody 1B10 to Eliminate Fibroblasts from Epithelial Cultures Figure 6 shows photomicrographs of a mixed culture of thymic epithelial cells and fibroblasts prior to 1B10 and complement treatment (Fig 6*A*), lysed fibroblasts following 1B10 + C′ treatment (Fig 6*B*), epithelial cells remaining immediately following treatment with 1B10 and C′ (Fig 6*C*), and the same cell culture 1 week following 1B10 and C′ treatment (Fig 6*D*). In order to assess quantitatively lysis of fibroblasts by antibody 1B10 plus complement, we trypsinized cell cultures and treated aliquots of cells with antibody 1B10 plus complement. Cells were then processed in indirect immunofluorescence for expression of 1B10 before and after treatment with antibody 1B10 plus complement and analyzed by cytofluorography. Treatment of cell suspensions with antibody 1B10 plus complement reduced the number of cells expressing cell surface 1B10 from 10.2% to 1.9%. An additional treatment with 1B10 plus complement did not further reduce 1B10 positive cell numbers (data not shown).

DISCUSSION

In this study, we report a monoclonal antibody (1B10) that fixes complement and binds and lyses human fibroblasts. We have used this antibody to reduce or eliminate fibroblasts in mixed cultures of human thymic epithelial cells and fibroblasts. Removal of fibroblasts using antibody 1B10 and complement was most efficient in young cultures where fibroblast growth was not yet extensive.

The antigen detected by antibody 1B10 was expressed on the surface of human foreskin fibroblasts, human thymic fibroblasts, and human synovial fibroblasts and thus did not appear to be differentially expressed on fibroblasts of different origin as has been described for other antigens [19]. Abbond et al have reported a monoclonal antibody reactive with human fibroblasts and endothelial cells but nonreactive with monocytes [20]. Human epithelial cells did not show surface expression of the antigen detected by 1B10. Interestingly, antibody 1B10 bound to human peripheral blood monocytes and tissue macrophages. Biochemical characterization indicated that antibody 1B10 binds to molecules of Mr 43,000 and 72–80,000. Cytotoxic antibodies have been raised to corneal fibroblasts and used to reduce fibroblasts in corneal epithelial cultures [21]. The reactivity of these antibodies with monocytes and macrophages was not addressed nor was the biochemical nature of the antigens detected. A review of the myeloid antibodies characterized in the Third International Leukocyte Differentiation Workshop suggested that antibody 1B10 does not fall into any of the known clusters of differentiation [22].

Antibody 1B10 should provide a powerful tool for control of fibroblast growth in a variety of human cell culture systems. Limited amounts of the antibody are available upon request for use by other investigators.

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